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TITLE OF THE INVENTION (280 characters max)

THE HETEROGENEOUS RIBONUCLEOPROTEIN K (hnRNP K) IS A REGULATORY COMPONENT IN HEPATITIS B VIRUS REPLICATION

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The Heterogeneous Ribonucleoprotein K (hnRNP K) is a Regulatory Component in Hepatitis B Virus Replication

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Hepatitis B virus (HBV) infection is endemic in many parts of Asia and Africa[1]; and in spite of the available vaccines[2-5] and anti-viral treatments[6], there remains, globally, a large number of hepatitis B carriers with a life-time risk for cirrhosis and hepatocellular carcinoma. Thus, understanding the dynamics of the HBV chronic infection state is of public health importance. The cellular mechanism of viral persistence, however is still poorly understood[7]. We studied the host-pathogen interactions in hepatitis B carriers by associating serum viral load and sequence variants in the recovered virus. We found a significant association between high patient viral load and a natural variant mutation within the HBV enhancer II (Enh II) regulatory region at position 1752. Furthermore, a host DNA-binding protein belonging to a class of heterogeneous nuclear ribonucleoproteins (hnRNP K), which have been implicated in diverse molecular and cellular functions, was shown to bind to this precise location of the virus. Finally, overexpression of hnRNP K in cell lines directly augmented HBV production. This is the most direct demonstration yet of the dependence of HBV on host proteins to modulate its steady-state replication and could represent a new class of targets for the control of HBV induced chronic disease.

HBV is the prototype for a family of viruses, referred to as *Hepadnaviridae* and is 3.2kb in size which encodes only four genes: the surface gene (*S*); the core gene (*C*); the X gene (*X*); and the polymerase gene (*P*). Although there is a large body of work on the biology of this virus and its association with cirrhosis and hepatocellular carcinoma, there is a paucity of information on how the virus directly engages the host[8-11]. This is especially so in the establishment of a chronic HBV infection. As many individuals infected during infancy still remain infectious carriers for decades leading to chronic hepatitis, a serious public health problem persists worldwide despite effective vaccination against HBV[1]. Host cellular factors play important roles in the HBV viral infectious life cycle[8-11] and identifying host factors that modulate viral replication in the chronic phase of HBV infection will be useful in controlling disease progression in chronic carriers.

To study the influence of HBV genotype on the resultant dynamics of viremia in HBV carriers, serum samples from 58 carriers were first analysed for viral load using a commercially available test kit (Digene). The serum viral load among HBV carriers was found to vary by more than 3 orders of magnitude (0.1-200 pg/ml) suggesting that there could be some mechanism such as antigen clearance or differential viral replication efficiency at work. The distribution almost appeared bimodal with about 60% (36/58) of the carriers had single digit pg/ml (range: 0.1-5 pg/ml) titer of viral DNA while 40% (22/58) had >10 pg/ml (range 11-199 pg/ml) titer (**Fig. 1a**). To determine if viral genotype had any link with viral load in the carriers, HBV DNA was amplified by PCR from serum, sequenced and aligned. Interestingly, within the entire viral genome, we observed a unique nucleotide position that showed significant correlation ($p=0.0001$) with viral load as defined by the two broadly partitioned groups of serum viral load described above (using 10 pg/ml as an arbitrary cutoff). As there was no particular physiological phenotype linked to a threshold HBV viral load, we also applied cutoff values of 20 pg/ml and 50 pg/ml; and in both instances, the

results were similar to the 10 pg/ml value. We observed a distinct tendency for carriers with high levels of serum HBV DNA to possess an A nucleotide at position 1752 of the virus sequence, while those with low levels of serum HBV DNA tend to have a G nucleotide at this position. This natural mutation at position 1752 was found to be located at the Enh II region of the HBV genome which has been shown in to be a specific regulator of HBV replication[8,9,11,12,13]

As this mutation resided in the viral enhancer element, we proceeded to study the effects of this base substitution on transcriptional efficiency by cloning a 131 bp Enh II fragment bearing 1752A upstream of a SV40 promoter-luciferase reporter gene (Promega). Site-directed mutagenesis was then carried out to generate three other constructs bearing 1752G, 1752T and 1752C so that four different constructs but differing from each other at only this single base nucleotide can be tested. Transient transfections were carried out on the hepatic cell line HepG2 and subsequently assayed for luciferase activity. The results showed that the construct with 1752A strongly enhanced the SV 40 promoter when linked to it in *cis* resulting in significantly higher levels (2-5 fold) of luciferase expression as compared to all other base substitutions (**Fig. 1c**). To determine if these results are specific only to the host HepG2 cell line, the transfections were repeated in three other hepatic cell lines: SKHep1, PLC/PRF/5 and HCCM. The results turned out to be consistent and reproducible, further substantiating that the single base change to an adenine at nucleotide position 1752 has a significant effect on the transcriptional efficiency of Enh II.

In order to further establish the biological significance of this point mutation observed in the Enh II region and to determine whether the luciferase assay results was merely epi-phenomena, we wanted to demonstrate the presence of any direct physical HBV DNA-host interaction at the position 1752 site. To this end, 28-mer oligonucleotide probes were designed to contain either an A or a G nucleotide (named Probes 2 and 4 respectively), and

two other probes of the same length (named Probes 1 and 3) were also designed using an irrelevant sequence slightly upstream of Probes 2 and 4, to function as controls. Electrophoretic mobility shift assays (EMSA) were performed using HepG2 nuclear extracts with the respective probes. A distinct DNA-binding protein was detected with Probe 2 (**Fig. 2a, lanes 5 to 8**) which has a nucleotide A at position 1752 and Probe 4 (**Fig. 2a, lanes 13 to 16**) with a nucleotide G at position 1752; no detection was observed from Probes 1 and 3 (**Fig. 2a, lanes 1 to 4, and lanes 9 to 12**). Densitometric analyses of bands indicated that the protein detected by Probe 2 was about 3 times higher than that detected from Probe 4, suggesting that position 1752A has a higher binding affinity of the DNA-binding protein to the Enh II region.

To further characterize this DNA-binding protein, nuclear extracts of HepG2 was passed through an affinity column tagged with the oligonucleotide-streptavidin binding probe (bearing 1752A) and the bound material were eluted and subjected two-dimensional (2-D) gel analysis. Specific protein spots were visualized by silver staining of approximately 56 kDa, showing positive enrichment of a specific DNA-binding protein compared with the non-specific binding control oligonucleotide probe. The spots were cut out and subjected to liquid chromatography/tandem mass spectrometry (LC/MS/MS) (**Fig. 2b**). The multiple peptide sequence results of the unknown DNA-binding protein showed significantly high sequence homology to hnRNP K **Fig. 3a**). The hnRNP K protein which belongs to the family of heterogeneous nuclear ribonucleoproteins (hnRNPs) is about 56 kD in size and has been implicated in diverse molecular and cellular functions, including nuclear-cytoplasmic shuttling[14] and transcription and translation[15]. hnRNP K has been shown to comprise multiple modular domains such as the K homology (KH) domains[16] and RGG boxes that allow it to interact with a diverse group of molecular partners like DNA and RNA[17-20]. The peptide sequence and molecular weight data provide strong support that the MS/MS analysis

has correctly identified the protein bound by the oligonucleotide-affinity column. Reports have indicated that interactions between hnRNP K and single-stranded DNA are mediated by three KH domains[16], and that hnRNP K exhibit specific binding and transactivation within the c-*myc* promoter[21-25]. hnRNP K was also shown to interact physically with proto-oncogenes like c-*src* and *vav*[17,18,26,27] thus allowing it to form multienzyme complexes and facilitate kinase cross-talk. This suggests that hnRNP K is a versatile molecule that can act as a docking platform for molecules involved in signal transduction and gene expression.

To demonstrate the functional connection of hnRNP K to the regulation of HBV replication, a full-length hnRNP K gene was cloned into the mammalian expression vector pcDNA 3.1 (Invitrogen). The hnRNP K expression construct was co-transfected into HepG2 cells together with a replicative clone of HBV[28] to determine the effect of hnRNPK on the replicative efficiency of the HBV construct. As expected, the HBV viral titer increased in a dose dependent manner with hnRNPK concentration (**Fig. 3b**). As a control, the empty expression vector pcDNA 3.1 did not have any effect on the HBV viral titer. Taken together, this set of co-transfection data strongly support the positive regulatory role of hnRNP K in HBV viral replication.

The work describe herein suggests that the clinical phenomenon of HBV viral load in carriers can be traced functionally to a single host protein. There are likely to be other factors that influence the interplay between the Enh II 1752 position with hnRNP K. There are possibilities such as hnRNP K polymorphisms[29], phosphorylation states (**Fig.** 2b; ref. 16, 17) and perhaps other cellular protein factors[14-27] that may act in concert to increase the regulatory replication activity of HBV in the host.

That hnRNP K binding is mapped precisely to a single base polymorphism in the viral genome's regulatory region suggests that an interesting dynamic exists between the genotype of the HBV at the Enh II region and genotypes that might modulate hnRNP K expression or

function in human populations. Indeed, a deeper understanding of how this works mechanistically will provide insights into defining virulence and fitness of a virus. Moreover, our work points to hnRNP K as a potential drugable target. Recently, cellular expression of hnRNP K can be downregulated by monoclonal anti-EGFR antibody[30] suggesting that modulation of EGRF by existing and approved pharmaceuticals may also alter HBV viral load in chronic carriers. Hence targeting host proteins could become an entire new paradigm for treatment of viral infections.

METHODS

Serological assays. Serum samples were aliquoted and stored at -20 °C until they were defrosted for testing. We examined serum HBV DNA levels by the Hybrid Capture II HBV DNA Assay (Digene)

Cells. Four cell lines, HCCM, HepG2, PLC/PRF/5 PP5 and Sk-Hep-1, derived from human hepatocellular carcinomas were used in this study. HCCM and PLC/PRF/5 PP5 contained copies of the integrated HBV genome, while HepG2 and Sk-Hep-1 were obtained from patients with no history of HBV infection and HBV genome integration[31-33]. Cells were cultured and maintained in complete Dulbecco's modified Eagle's medium (Invitrogen) and supplemented with 10% foetal bovine serum (Cytosystems) at 37 °C in humidified 5% CO_2.

Gel-shift assays (EMSA). Binding reaction procedures were performed at 37 °C for 20 min in 20 µl reaction mixtures (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA and 1 mM DTT) containing 10 µg of HepG2 nuclear extracts, 0.1 - 0.2 µg of non-specific competitor DNA poly (dI-dC) (Amersham Pharmacia Biotech, USA) and ^{32}P- dATP end-labelled probe (1×10^4 to 1×10^5 cpm). Free DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels. Gels were dried down under vacuum at 80 °C for 1 h before exposure to X-ray film (Biomax, Kodak) at -80 °C. The sequences of the oligonucleotide probes (nucleotide changes are indicated) were:

Probe 1: AGACTGTGTGTTTAATGAGTGGGAGGAG,

Probe 2: AGTTGGGGGAGGAGATTAGGTTAAAGGT

Probe 3: AGACTGTGTGTTTAATGCGTGGGAGGAG

Probe 4: AGTTGGGGGAGGAGGTTAGGTTAAAGGT

Transfection and Luciferase Assays. Cells were plated at an average seeding density of 5 x 10^4 cells per well in 24-wells plates and transfected with Gene Porter (Gene Therapy) according to manufacturer's instructions. Briefly, 3 μg of plasmid DNA were used for each transfection mix, and added dropwise onto the cells. After incubation for 48 h at 37 °C, the cells were subsequently harvested followed by genomic DNA extraction with the DNeasy Kit (Qiagen). HBV viral titre loads were measured as described (Digene). For the luciferase assays, 3 μg of plasmid DNA together with 1 μg of control / promoter luciferase plasmid-DNA were used for each transfection mix and after incubation for 48 h at 37 °C, cells were harvested with Cell Culture Lysis Reagent (CCLR; Promega). 20 μl of cell lysates were mixed with 100 μl of Luciferase Assay Reagent (Promega) and luciferase activity was measured as relative light units (RLU) determined with a Turner 20 / 20 luminometer (Promega). Relative luciferase activity was expressed as fold increase over vector without the Enhancer element. To control for variations in transfection efficiency, the experiments were performed in triplicates and repeated at least three times.

Affinity capture of host-interacting proteins, 2-D gel electrophoresis and protein identification. Nuclear protein extracts were obtained from HepG2 cells. HepG2 cells were harvested and rinsed twice with ice-cold buffer A (0.15 M NaCl, 10 mM HEPES, pH 7.4), and incubated on ice for 15 min with 5 x original packed cell volume of buffer B (0.33 M sucrose, 10 mM HEPES, 1 mM $MgCl_2$, 0.1% Triton X-100, pH 7.4). After centrifugation at 3,000 rpm for 5 min at 4 °C, the pellet was washed once with buffer B and resuspended gently on ice with 200 μl of buffer C [0.45 M NaCl, 10 mM HEPES, pH 7.4, with protease inhibitor cocktail (Sigma P8340)]. The cell mixture was incubated for 15 min with gentle agitation followed by centrifugation at 13,000 rpm for 5 min. The supernatant was saved for DNA-binding proteins assay. Annealing of double-stranded oligonucleotides probes were done using 100 μl of deionised Milli Q water containing 1 nmole each of anti-sense probe and

sense probe which were labelled with biotin at the 3' end, and 5' end, respectively. Oligonucleotide mixture solutions were heated at 95 °C for 5 min and cooled slowly to room temperature. DNA-interacting proteins were captured as described before[34]. Briefly, oligonucleotides mixture was incubated with 5 mg Dynabeads® M-280 streptavidin (Dynal Biotech) at room temperature for 15 min in binding and washing buffer (5 mM Tris-HCl, 0.5 mM EDTA, 1.0 M NaCl, pH 7.5). The magnetic beads were then washed with binding and washing buffer and equilibrated with TGED buffer (20 mM Tris-HCl, 10% glycerol, 1 mM DTT, 0.01% Triton X-100, 50 mM NaCl, pH 8.0). 40 μg of extracted nuclear proteins was mixed 2:1 (w/w) with non-specific competitor DNA poly (dI-dC) (Amersham Biosciences), and adjusted to 500 μl with TGED buffer. Unbound proteins were washed out with TGED buffer and bound proteins were eluted with TGED buffer with 1 M NaCl and subjected to acetone precipitation. We performed 2-D gel electrophoresis according to the Amersham Bioscience protocol with some modifications. Briefly, each sample containing acetone precipitated proteins was made up to a volume of 350 μl with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer pH 3 - 10, 1.0 mg of DTT). The mixture was mixed briefly by vortexing and centrifuged at 13,000 rpm for 10 min. The supernatant was loaded to 18 cm, pH 3 - 10 nonlinear Immobiline DryStrips and rehydration was carried out actively at constant voltage (50 V) overnight. Isoelectric focusing (IEF) was performed using IPGphor (Amersham Biosciences) at 20 °C in stepwise mode. Briefly, strips were focused at 500 V for 1 h, 2000 V for 1 h, 5000 V for 1 h, and 8000 V for 12 h, with a total of 90 KVh accumulated. After IEF, the IPG strips were incubated for 30 min in 15ml of SDS equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, 66 mM DTT, trace amount bromophenol blue, pH 8.8), followed by second incubation with the same buffer for 30 min with iodoacetamide (375 mg / 15 ml) instead of DTT. Second dimensional vertical SDS-PAGE (Protein II XL, Bio-Rad Laboratories) was carried out using 10% gels at a

constant voltage of 150 V for 6 - 8 h at 15 °C. Gels were stained with Silver staining kit (SilverQuest Silver Staining Kit, Invitrogen). Specific protein spots were cut out and destained according to manufacturer's instructions, following which the gel plug was dried and MS/MS analysis carried out by Proteomic Research Services, Inc.

Statistics. We compared the frequencies of the mutations among groups using Fisher's exact probability test.

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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Supplementary Methods

PCR and sequencing of the Enh II / pre-core region. DNA extracted from serum was amplified with primers 5'-TTCACCTCTGCACGTCGC-3' and 5'–GCTTGGAGGCTTGAACAG-3' (94 °C for 2 min, followed by 35 cycles of 94 °C for 15 sec, 50 °C for 30 sec, and 72 °C for 1 min and lastly followed by 72 °C for 7 min) with *Taq* DNA polymerase (Roche). Nested primers 5'–GTCAACGACCGACCTTGAGG-3' and 5'-ACCAATTTATGCCTACAGCCTC-3' were used in a second round of PCR (94 °C for 2 min, followed by 35 cycles of 94 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 1 min and lastly followed by 72 °C for 7 min). The size of this nested PCR product was 116 bp (corresponding to nucleotides 1682- 1798) and was resolved in 1.5 – 2% agarose gels. PCR products were cleaned up using the Qiaquick PCR Purification Kit (Qiagen) and sequenced directly to confirm the identity of the products using the ABI prism dRodamine Terminator Cycle Sequencing Ready Reaction Kit (ABI-Prism 310, Applied Biosystems). Results of the sequences were aligned and compared.

Construction of plasmids. Plasmids pGL3-Control (a Luciferase plasmid with a simian virus 40 enhancer and promoter) and pGL3-Promoter (an enhancerless Luciferase plasmid with a simian virus 40 promoter upstream from the Luciferase gene) were obtained from Promega. Plasmid pGL3-Promo / A was constructed by amplifying the basic functional unit of EnhII by PCR using primers LucF (5'–*GCACGCGT*CAACGACCGACCTTGAGG-3') and LucR (5'–*GCAGATCT*ACCAATTTATGCCTACAGCCTC-3') comprising HBV nucleotide positions 1686 to 1801. The 131 bp PCR fragment was *Mlu* I / *Bgl* II-digested and ligated with *Mlu* I / *Bgl* II-digested pGL3-Promoter. The other mutant constructs were constructed using the Gene Editor Site-Directed *in vitro* Mutagenesis System (Promega) to introduce the HBV Enhancer II mutations at nucleotide position 1752. The first mutation was mutating

nucleotide A to G (pGL3Promo / G), the second was with nucleotide A to T (pGL3Promo / T), and lastly with nucleotide A to C (pGL3Promo / C). The sequences of the three mutant oligonucleotides were: 5'–GGGGGAGGAGGTTAGGTTAAA-3', 5'–GGGGGAGGAGTTTAGGTTAAA-3', and 5'–GGGGGAGGAGCTTAGGTTAAA-3' respectively. Constructs were sequenced for verification. hnRNPK clone was constructed by cloning a 1.4-kb RT-PCR fragment coding for the hnRNPK from total RNA extracted from HepG 2 cells. The *EcoR* I- and *Xho* I-digested PCR fragment was cloned into *EcoR* I- and *Xho* I-digested pcDNA3.1.

The cloning primers were 5'–TAAAAGGAATTCAATATGCAAACTGAACAG–3' and 5'-CTAGTCCTCGAGTTAGAAAAACTTTCCAGA–3'. Sequencing was done for verification of the constructs.

Preparation of nuclear protein extracts. Cultures were trypsinized, rinsed twice with ice-cold 1x phosphate-buffered saline (PBS), and incubated on ice for 10 min with 5 x original packed cell volume (PCV) of buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N*'-ethane sulfonic acid (HEPES) buffer (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM dithiothreitol (DTT)]. After centrifugation at 1,000 rpm for 3 min at 4 °C, cells were resuspended in 2 x original PCV of buffer A and homogenized in a Dounce homogenizer with a S pestle with 10 strokes. Nuclei fractions were sedimented by a 10 min centrifugation at 2,500 rpm, resuspended in 1.5 x buffer B [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 25% Glycerol) and treated with another 10 strokes of Dounce homogenizer. Cell suspensions were then transferred to microcentrifuge tubes and incubated for 30 min at 4 °C with gentle rotation. Nuclear debris was removed by centrifugation at 13,000 rpm for 40 min at 4 °C. The supernatant was dialysed for 4 h against 2 changes of 200 ml Buffer C [20 mM HEPES pH 7.9, 0.2 mM EDTA, 20 mM $MgCl_2$, 20 mM KCl, 420

mM NaCl, , 25% Glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] at 4 °C. After dialysis, nuclear extracts were clarified by centrifugation at 13,000 rpm for 20 min. Nuclear extracts were then aliquoted and stored at -70 °C. Protein concentration was quantitated with the Protein Assay kit (Bio–Rad Laboratories) using acetylated bovine serum albumin as standard.

FIGURE LEGENDS

Figure 1 Distinct segregation between high and low viraemic HBV individuals is correlated to changes at nucleotide position 1752.

(a) Differences in the HBV viral load of HBV carriers. Data of the corresponding DNA sequences from nucleotide 1720 to 1760 to the HBV DNA titre levels of the subjects is illustrated. A total of 58 subjects were collated and DNA was isolated from sera, and sequenced.

(b) The Enh II is located just upstream of the core promoter. The minimum sequence for enhancer activity has been previously defined at nucleotide 1687-1805 as shown, and it has been shown to be involved in viral replication.

(c) Effect of nucleotide 1752 base substitution on Enh II activity. Cells were transfected with the respective Enh II clones (1752A, 1752G, 1752T and 1752C), harvested at 48 h p.t., and followed by luciferase activity analysis. Results of the luciferase assay were normalized to the level of pGL3Control (arbitrarily set at 100%). For each cell type, the first column represents the luciferase activity of the internal positive control (promoter and enhancer). The second column represents the activity of the internal negative control (promoter only). The other columns of each cell line represent the vectors with the promoter and the wild-type or mutated Enh II (nucleotide 1752A, 1752G, 1752T, or 1752C).

Figure 2 Evidence of the involvement of a host cellular protein in Enh II activity.

(a) EMSA was performed using HepG2 nuclear extracts with four different probes. Probe 1 (A^{1727}): Lanes 1 to 4; Probe 2 (A^{1752}): Lanes 5 to 8; Probe 3 (C^{1727}): Lanes 9 to 12; (G^{1752}): Lanes 13 to 16. Each set of probes contains increasing concentrations

(0.0 μg, 0.05 μg, 0.10 μg and 0.15 μg) of non-specific competitor DNA [poly-(dI)-poly-(dC)] of [poly-(dI)-poly-(dC)] respectively.

(b) First-dimensional isoelectric focusing (18 cm, pH 3 to 10 strip) of the DNA-binding protein, followed by separation on SDS-PAGE (10 %). The estimated molecular weight of the specific protein spots detected by silver staining (arrow) is indicated.

Figure 3 Detection of cellular protein as hnRNPK and its involvement in HBV replication.

(a) Results of peptide sequencing of the 56 kDa protein by LC/MS/MS showed high homology scores to hnRNPK in sequence alignments.

(b) HepG2 cells were transfected with full-length infectious replicative HBV, hnRNPK and pcDNA3.1. Cells were harvested at 48 p.t. and HBV DNA viral load was measured. "+" and "++" indicates 3 μg and 6 μg of plasmid transfected DNA respectively.

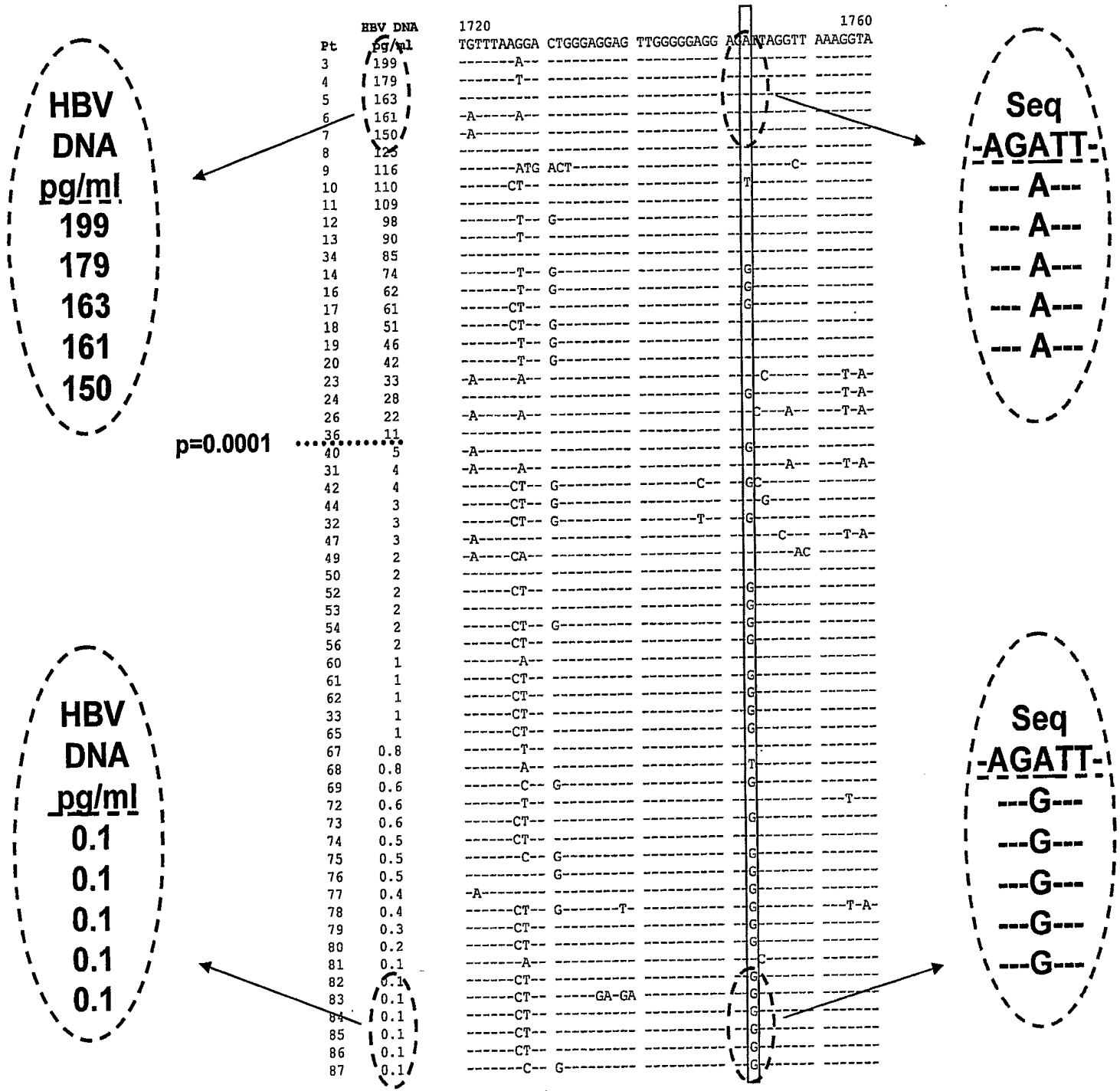


Fig. 1a

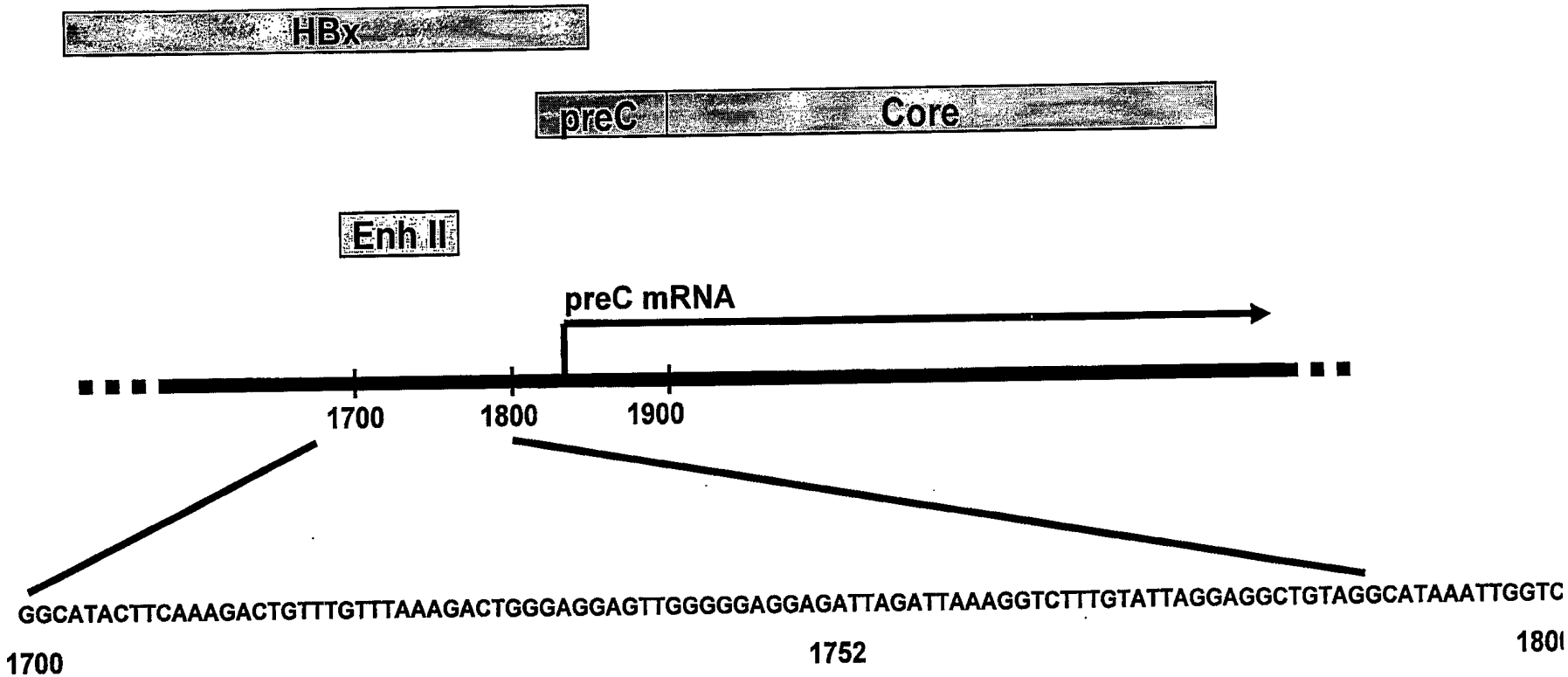


Fig. 1b

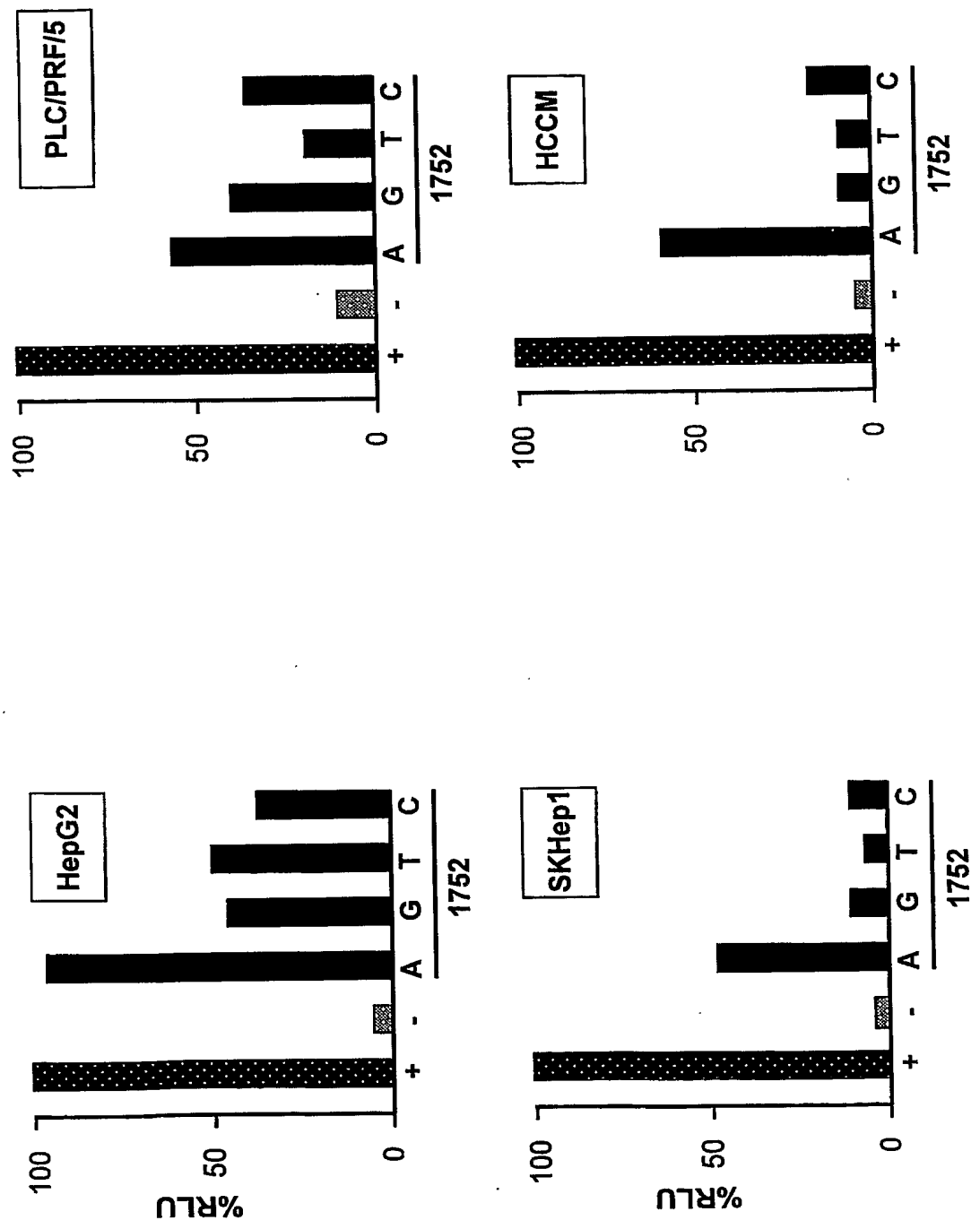
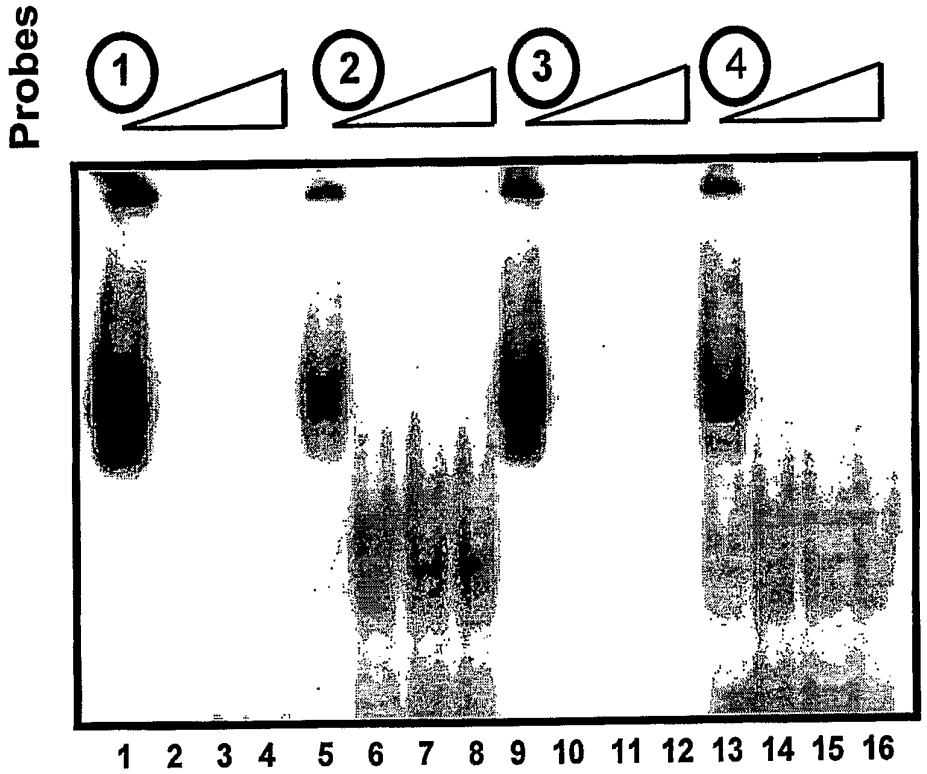


Fig. 1c



① AGACTGTGTGTTTAATGAGTGGGAGGAG

② AGTTGGGGGAGGAGATTAGGTTAAAGGT

③ AGACTGTGTGTTTACTGCGTGGGAGGAG

④ AGTTGGGGGAGGAGGTTAGGTTAAAGGT

GGCATACTTCAAAGACTGTTTGTTTAATGACTGGGAGGAGTTGGGGGAGGAGATTAGATTAAAGGTCTTTGTATTAGGAGGCTGTAGGCATAAATTGGTC

1700 1752 180

Fig. 2a

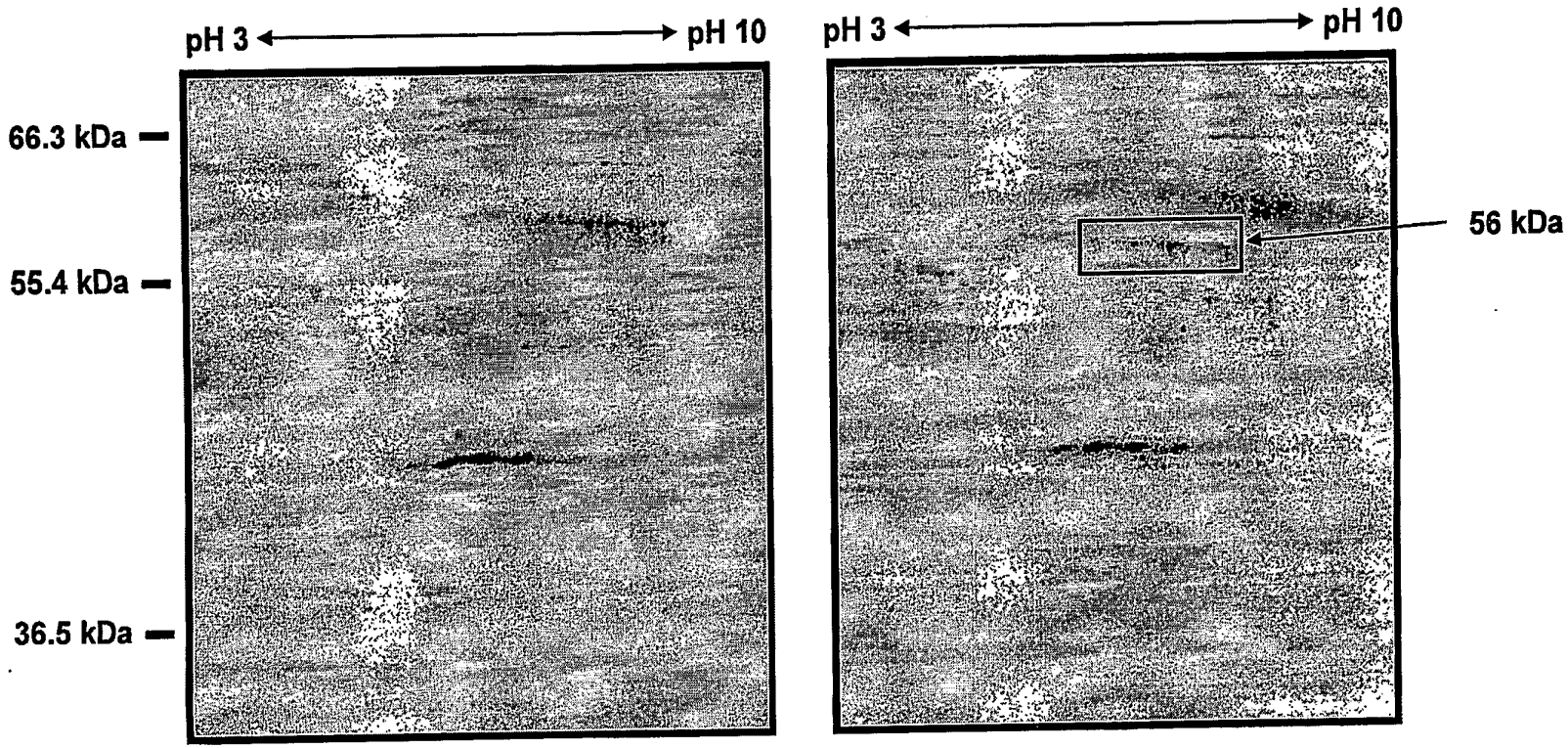


Fig. 2b

Fig. 3a

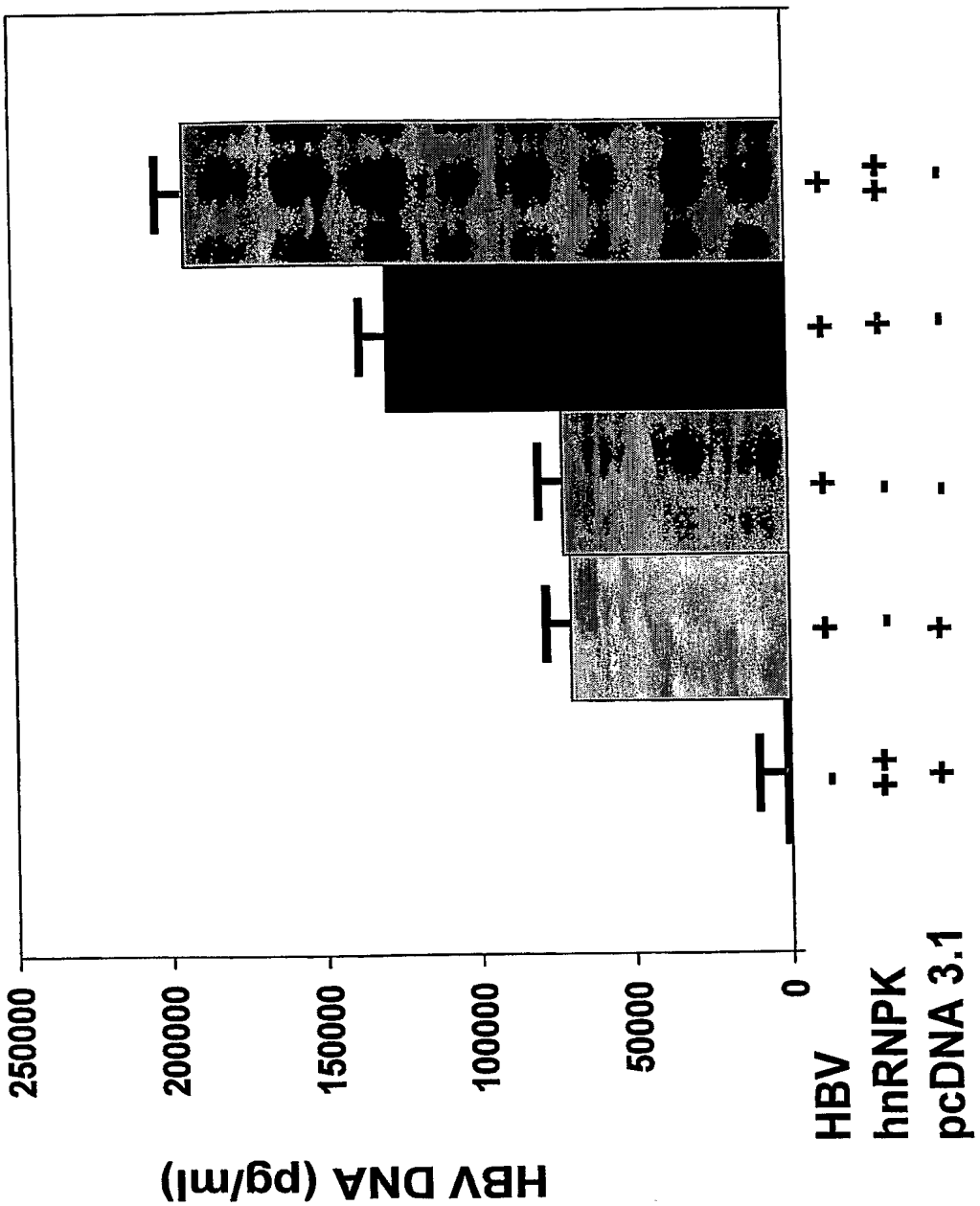


Fig. 3b